The Utilization of Nitriles and Amides by *Nocardia rhodochrous*

By PAULINE A. COLLINS AND CHRISTOPHER J. KNOWLES*

Biological Laboratory, University of Kent, Canterbury, Kent CT2 7NJ, U.K.

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Nocardia rhodochrous LL100-21 grew on acetonitrile, acetamide, a range of aliphatic nitriles and amides, benzonitrile and benzamide as the sources of carbon and/or nitrogen. It also grew on acetate, several other aliphatic acids, and benzoate as carbon sources. Growth on either acetonitrile or acetamide resulted in induction of both acetonitrilase and acetamidase activities, which were shown to be due to separate enzymes. Growth on benzonitrile resulted in the ability to release ammonia from benzonitrile but not benzamide, acetonitrile or acetamide. Growth on benzamide resulted in the ability to form ammonia from benzamide but not benzonitrile, acetonitrile or acetamide. Measurements of respiratory activity of the bacteria grown on the various substrates showed a similar pattern of induction of oxidative activity for these compounds.

INTRODUCTION

Relatively little is known about the ability of micro-organisms to utilize nitriles as carbon and/or nitrogen sources (Jallageas *et al.*, 1980; Chamberlain & MacKenzie, 1981). Aliphatic nitriles are catabolized in two stages, via conversion to the corresponding amide and then to the acid plus ammonia, by a nitrilase and an amidase respectively (Arnaud *et al.*, 1976*a, b*, 1977; Jallageas *et al.*, 1978; DiGeronimo & Antoine, 1976; Asano *et al.*, 1980). The utilization of amides by micro-organisms has been extensively studied, particularly in *Pseudomonas aeruginosa* (Clarke, 1980) which is, however, unable to utilize simple aliphatic nitriles (J. M. Miller & C. J. Knowles, unpublished observations). Benzonitrile and related aromatic nitriles (Harper, 1976, 1977*a, b*) and an heterocyclic nitrile (Robinson & Hook, 1964; Hook & Robinson, 1964) have been shown to be converted directly to the corresponding acids and ammonia with little release of the amide as an intermediate. The nitrilases were purified, and in both cases there was no separate amidase required for formation of the acid product.

In order to learn more about the ability of bacteria to utilize nitriles as carbon and nitrogen sources we have investigated the growth of *Nocardia rhodochrous* LL100-21 on acetonitrile and benzonitrile and the related amides and acids. This organism was originally isolated by DiGeronimo & Antoine (1976) and shown to utilize aliphatic nitriles.

METHODS

Organism and growth conditions. *Nocardia rhodochrous* strain LL100-21 (supplied by Dr A. D. Antoine, Rutgers University) was grown on a minimal medium consisting of M-9 salts (Miller, 1972) with ammonium salts omitted, 1 ml trace metals l⁻¹ (Bauchop & Elsdon, 1960) and the following carbon and nitrogen sources: acetonitrile (25 mM), acetamide (20 mM), acetate (20 mM) plus NH₄Cl (20 mM), benzonitrile (6 mM), benzamide (6 mM), benzoate (6 mM) plus NH₄Cl (20 mM). Other carbon and nitrogen sources and the concentrations used are given in Table 1. A 2% (v/v) inoculum of a 20 to 24 h stationary phase culture grown on the same carbon and nitrogen sources as the experimental culture was used to inoculate the experimental flasks. Growth was in either 50 ml medium in 250 ml conical flasks or 500 ml medium in 2 litre conical flasks. Incubation was at 30 °C in an orbital shaker (250 r.p.m.).

Harvesting and preparation of cell-free extracts. Bacteria were harvested in the mid-exponential phase (A₇₅₀ 0.3 to 0.8) by centrifugation at 23000 g for 10 min at 4 °C, washed twice in 100 mM-KH₂PO₄/K₂HPO₄ buffer (pH 7.0)

and resuspended in fresh buffer to about one-twentieth of the original culture volume. Bacteria were disrupted by sonication (MSE sonicator, 150 W, 6×15 s, 0°C) and centrifuged at 10000 g for 10 min at 4°C to give a cell-free extract. Where indicated, this was centrifuged at 150000 g for 90 min at 4°C to separate it into particulate and supernatant fractions. The particulate fraction was resuspended in 100 mM-phosphate buffer (pH 7.0).

Determination of respiratory activity. Oxygen uptake by intact bacteria was measured at 30°C with an oxygen electrode (Rank Bros., Bottisham, Cambridge, U.K.). The incubation mixture contained 0.3 to 0.8 mg dry wt bacteria in 100 mM- $\text{KH}_2\text{PO}_4/\text{K}_2\text{HPO}_4$ buffer (pH 7.0), in a total volume of 2.9 ml. The reaction was initiated by addition of 0.1 ml of 300 mM substrate. In the case of benzamide and benzonitrile, which are of low solubility, the reaction was initiated by adding 0.1 ml bacteria to 2.9 ml buffer containing 10.7 mM-benzamide or benzonitrile.

Determination of ammonia, acetamide, acetonitrile and acetate in the growth medium. Samples of the growth medium (4 ml) from cultures containing acetonitrile or acetamide were centrifuged for 1 min in a Microfuge (Beckman Instruments Inc., Palo Alto, Calif., USA). From the supernatant a 100 μl portion was frozen overnight at -20°C prior to GLC analysis and a second 10 μl sample mixed with 1 ml nitroprusside reagent (Fawcett & Scott, 1960), stored overnight at -20°C and then assayed for ammonia. For GLC analysis the 100 μl portion was thawed and mixed with 25 μl 25 mM-propionic acid (as an internal standard) in 250 mM-HCl, and a 2 μl sample injected into a model 204 gas chromatograph (Pye Unicam Ltd., Cambridge) equipped with a flame ionization detector and linked to a model CDPI integrator. A glass column (1.5 m by 4 mm i.d.) was packed with Porapak Q (Waters Association, Inc., Milford, Mass., USA), mesh 80–100. The operating temperature for the detector was 250°C , for the column 190°C , and for the injector 210°C . The carrier gas was N_2 at 50 ml min^{-1} . Quantitative measurements were obtained by comparison with a standard curve of known concentrations of the test compounds by their peak areas relative to that of the propionate internal standard. For ammonia analysis the sample that had been mixed with nitroprusside reagent was thawed and mixed with 1 ml alkaline hypochlorite reagent (Fawcett & Scott, 1960). The solution was then incubated at 35°C for 15 min and the A_{570} measured. An NH_4Cl solution was used as a standard. The acetamide content of the medium was also estimated by colorimetric analysis (Snell & Snell, 1954), with good agreement with the GLC analysis.

Determination of nitrilase and amidase activities. Acetamidase activity was routinely measured by GLC analysis of the acetate formed or by colorimetric assay of the ammonia produced. Because acetamidase activity was lower than acetonitrilase activity, the latter could not be assayed simply via acetate or ammonia formation. Acetonitrilase activity was therefore measured by GLC analysis of acetonitrile disappearance or, routinely, as the sum of the initial rates of acetamide and acetate formation; preliminary colorimetric measurements of ammonia formation showed that it appeared at the same rate as acetate. Benzonitrilase and benzamidase activities were assayed colorimetrically by the ammonia released. The reaction mixture (1 to 10 ml) at 30°C contained 50 mM- $\text{KH}_2\text{PO}_4/\text{K}_2\text{HPO}_4$ buffer (pH 7.0, except for acetamidase which was at pH 6.5), 25 mM or 5 mM-acetonitrile or acetamide, or 5 mM-benzonitrile or benzamide, and bacteria, cell-free extract, particulate or supernatant fraction. The reaction was initiated by addition of substrate for the assays with aliphatic compounds and of bacteria or extract for the assays with the less soluble aromatic compounds. For GLC analysis of the aliphatic nitrilase and amidase reaction products 100 μl samples of the reaction mixture were removed at intervals up to 30 min and added to 25 μl of 50 mM-propionic acid in 250 mM-HCl. When intact bacteria were used they were removed by centrifugation for 30 s in a Microfuge. The samples were stored at -20°C until required. They were then assayed by GLC as described above, except that when the substrate was acetamide the column was at 210°C and the injector at 230°C . Ammonia was assayed by the method of Fawcett & Scott (1960).

Other assays. Protein was measured by the modified biuret method of Gornall *et al.* (1949). Bacterial growth was measured by the A_{750} .

Chemicals. Acetamide, acetonitrile, benzamide, benzoic acid, formamide, biotin, vitamin B_{12} and hydroxylamine were obtained from Sigma. Benzonitrile and acetic acid were obtained from BDH. Acetamide and benzamide were recrystallized from hot ethanol, and benzoic acid from hot water. Wherever possible, all other reagents were of analytical grade. Glass-distilled water and acid-washed glassware were used throughout.

RESULTS

The ability of *N. rhodochrous* strain LL100-21 to grow on a range of nitriles and the corresponding amides and acids is shown in Table 1, which extends the studies of DiGeronimo & Antoine (1976). The one-carbon compounds cyanide, formate and formamide were unable to act as carbon sources for growth although formamide, but not cyanide, served as a nitrogen source. A range of simple aliphatic nitriles and amides acted as carbon and/or nitrogen sources. Growth also occurred when the corresponding acids were used as the carbon source. The unsaturated aliphatic nitriles and amides supported growth only when provided as the source of nitrogen. Addition of a phenyl group to the aliphatic side-chain (phenylacetamide and

Table 1. Carbon and nitrogen sources for growth of *N. rhodochrous* LL100-21

An A_{750} of at least 1.0 after 24 h growth is indicated by +, slower growth to an A_{750} of at least 1.0 after 72 h by +(S), and no growth (A_{750} less than 0.05) 96 h after inoculation by —.

Compound	Concn (mM)	As C and N source	As C source (with 20 mM- NH_4Cl)	As N source (with 10 mM-succinate)
KCN	2 & 20	—	—	—
Formate	20	—	—	—
Formamide	20	—	—	+
Acetate	20	—	+	—
Acetamide	20	+	+	+
Acetonitrile	25	+	+	+
Propionate	20	—	+	—
Propionamide	20	+	+	+
Propionitrile	20	+	+	+
Butyrate	20	—	+(S)	—
Butyramide	20	+(S)	+(S)	+
Valerate	20	—	+(S)	—
Valeronitrile	20	+	+	+
Acrylate	6	—	—	—
Acrylamide	6	—	—	+
Acrylonitrile	6	—	—	+
Methacrylamide	6	—	—	+
Methacrylonitrile	6	—	—	+
Phenylacetamide	20	—	—	—
Phenylacetonitrile	20	—	—	—
Benzoate	6	—	+	—
Benzamide	6	+	+	+
Benzonitrile	6	+	+	+
Lactate	12	—	+	—
Succinate	10	—	+	—

phenylacetonitrile) resulted in no growth on the compounds as either carbon or nitrogen sources. Unlike DiGeronimo & Antoine (1976), we obtained rapid and profuse growth with benzonitrile as the carbon and/or nitrogen source. This may be due to the lower concentration of this compound used in our study (2 mM or 6 mM, compared to about 100 mM). The bacterium also grew well on benzamide as a carbon and/or nitrogen source.

During growth on acetamide as the sole source of carbon and nitrogen, substantial amounts of ammonia and acetate appeared in the growth medium (Fig. 1*a*). Growth terminated due to acetate depletion from the medium. Growth on acetonitrile was accompanied by the sequential formation of acetamide and acetate plus ammonia in the culture medium (Fig. 1*b*). Growth terminated due to acetate depletion from the medium.

The rates of respiration by intact harvested bacteria that had been grown under various conditions are shown in Table 2. Bacteria that had been grown with succinate as the carbon and energy source oxidized succinate and acetate at a high rate but had little ability to oxidize acetamide, acetonitrile, benzoate, benzamide or benzonitrile. Bacteria grown under all the other conditions oxidized succinate at a lower rate. Bacteria grown on succinate plus formamide oxidized formamide at only a relatively low rate.

Bacteria that had been grown on acetonitrile or acetamide rapidly oxidized both these substrates as well as acetate, whereas acetate-grown bacteria oxidized acetate but not acetamide or acetonitrile. Bacteria that had been grown on acetate, acetamide or acetonitrile showed little activity with benzoate, benzamide or benzonitrile. Bacteria that had been grown on benzonitrile oxidized benzonitrile and benzoate at a high rate but had little capacity to oxidize benzamide, whereas bacteria that had been grown on benzamide oxidized benzamide and benzoate much more rapidly than benzonitrile. It should be noted that an initial lag was observed for oxidation of benzonitrile or benzamide but not benzoate by benzamide- or benzonitrile-grown bacteria. Bacteria grown on benzoate oxidized it and also to a certain extent benzamide but not

Fig. 1. *N. rhodochrous* was grown on 20 mM-acetamide (a) or 25 mM-acetonitrile (b). Samples (3 ml) were aseptically removed from the cultures and growth was measured as A_{750} (●). Samples (4 ml) were centrifuged for 1 min in a Microfuge to remove bacteria. The ammonia concentration in the medium (○) was measured colorimetrically, and the acetonitrile (□), acetamide (▲) and acetate (■) concentrations were determined by GLC.

benzonitrile. Bacteria grown on benzonitrile, benzamide or benzoate showed little oxygen uptake with acetonitrile, acetamide, acetate or formamide.

Acetonitrilase and acetamidase activities of bacteria grown on acetonitrile, acetamide and acetate were measured in intact cells, extracts, and particulate and supernatant fractions (Table 3). Both enzymes were formed by bacteria grown on either acetonitrile or acetamide but were not induced by growth on acetate. Both enzymes were found in the supernatant fraction obtained by centrifugation of extracts at 150 000 g for 90 min. It is also noteworthy that activity was seen for both enzymes with intact cells as well as extracts. Bacteria grown on acetate, acetamide or acetonitrile, or extracts derived from them, were unable to release ammonia from benzonitrile or benzamide.

Acetamidase and acetonitrilase activities were shown to be due to separate enzymes. Experiments with cell-free extracts showed that acetamidase had a broad pH optimum at 7.0, with 80% of maximal activity at pH 6.0 and 8.0. In contrast, acetonitrilase had a sharp pH optimum at 6.5 and only 50% of maximal activity at pH 7.0. Addition of 2 mM-EDTA or 100 μ g pyridoxal phosphate ml^{-1} to extracts caused partial inhibition of acetonitrilase activity but had no effect on acetamidase activity. No stimulation of the activity of either enzyme was observed with 100 μ g biotin ml^{-1} , 1 μ M-vitamin B_{12} or 3 mM- MgSO_4 . The inhibitory effects of EDTA and pyridoxal phosphate on acetonitrilase were probably due to their ability to chelate metals, whereas the lack of stimulation by MgSO_4 suggested that there was a sufficient concentration of divalent metals in the extract to maximally stimulate the enzyme. This was confirmed by dialysis of the extract overnight at 4 °C against 5 mM- $\text{NaH}_2\text{PO}_4/\text{Na}_2\text{HPO}_4$ buffer (pH 6.5) containing 3 mM-EDTA. Acetonitrilase activity was completely lost whereas acetamidase was unaffected.

Table 2. *Oxygen uptake by N. rhodochrous that had been grown on various substrates*

The values for oxygen uptake are for the initial rates except for benzamide and benzonitrile, where there was an initial lag period prior to linear uptake.

Substrate tested for oxidase activity	O ₂ uptake [$\mu\text{g atom min}^{-1} (\text{mg dry wt})^{-1}$] after growth on:						
	Acetonitrile	Acetamide	Acetate + NH ₄ Cl	Benzonitrile	Benzamide	Benzoate + NH ₄ Cl	Succinate + formamide
Acetonitrile	174	255	0	2	6	32	21
Acetamide	205	272	11	8	7	33	9
Acetate	230	248	239	18	27	12	69
Benzonitrile	0	0	6	160	58	0	7
Benzamide	20	0	7	24	201	52	19
Benzoate	0	11	11	175	197	95	12
Formamide	0	0	0	4	5	6	19
Formate	0	0	11	0	8	0	16
Succinate	0	7	13	16	12	8	109

Table 3. *Distribution of acetonitrilase and acetamidase activities in N. rhodochrous grown on acetonitrile, acetamide and acetate*

Enzyme activities were measured at 30 °C in intact cells, extracts, and particulate and supernatant fractions of bacteria that had been harvested 12 h after inoculation and in the mid-exponential growth phase (A_{750} 0.4 to 0.7). Acetonitrilase activity was measured by the initial rate of formation of acetamide plus acetate, and acetamidase by the initial rate of formation of acetate.

Growth substrate	Enzyme studied	Enzyme activity [$\text{mmol min}^{-1} (\text{mg protein})^{-1}$]			
		Whole cells	Cell-free extract	Supernatant fraction	Particulate fraction
Acetonitrile	Acetonitrilase	1.77	1.94	3.27	0.09
Acetonitrile	Acetamidase	0.22	0.24	0.53	0.03
Acetamide	Acetonitrilase	1.04	0.59	1.29	0.02
Acetamide	Acetamidase	0.30	0.53	0.86	0.02
Acetate	Acetonitrilase	0	0	0	0
Acetate	Acetamidase	0	0	0	0

Addition of 3 mM-MgSO₄ or 3 mM-MnSO₄ to an assay solution containing 50 mM-NaH₂PO₄/Na₂HPO₄ buffer (pH 6.5) and 0.3 mM-EDTA restored the acetonitrilase activity. Treatment of the supernatant fraction with (NH₄)₂SO₄ resulted in separation of the nitrilase and amidase. Acetamidase was located almost entirely in the 55 to 80% saturated (NH₄)₂SO₄ fraction whereas acetonitrilase was totally in the 40 to 55% fraction.

Bacteria grown to the mid-exponential phase on 6 mM-benzonitrile formed ammonia from benzonitrile but not from benzamide. The benzonitrilase activity was 64 $\mu\text{mol ammonia formed min}^{-1} (\text{mg dry wt})^{-1}$ and, like oxygen uptake with benzonitrile as the substrate, was evident only after an initial lag phase. Ammonia was also formed from benzonitrile by extracts of benzonitrile-grown bacteria but again only after an initial lag period (see Harper, 1976, 1977*a*). The activity was then linear and was 28 $\mu\text{mol ammonia formed min}^{-1} (\text{mg protein})^{-1}$. The activity was located almost entirely in the 150000 g supernatant fraction. The nitrilase had a pH optimum in extracts at 7.0 to 7.4, with little activity at pH 6.5 and 8.0. Bacteria grown on 6 mM-benzamide formed ammonia from benzonitrile and benzamide after an initial lag period with activities of 25 and 53 $\mu\text{mol min}^{-1} (\text{mg dry wt})^{-1}$. Little benzonitrilase activity was observed in extracts. We have been unable to obtain any benzamidase activity in extracts of bacteria grown on benzamide, benzonitrile or benzoate when tested over a wide range of pH values (5.6 to 8.0). This may have been due to lability of the enzyme, or a requirement for a cofactor, etc., that we

have been unable to detect. Bacteria grown on benzoate formed less than 5 $\mu\text{mol ammonia min}^{-1}$ (mg dry wt^{-1}) from benzonitrile or benzamide, and benzonitrilase activity was not found in extracts. Bacteria grown on benzonitrile, benzamide or benzoate did not release ammonia from acetonitrile, acetamide or formamide.

DISCUSSION

Brevibacterium strain R312 and several other bacteria have been shown to form nitrilases and amidases of very broad substrate specificities (Arnaud *et al.*, 1976*a, b*, 1977; Jallageas *et al.*, 1978). However, *Pseudomonas aeruginosa* and several other micro-organisms form amidases that utilize only a small range of aliphatic amides (Clarke, 1980). These amidases, where studied, have been shown to be inducible. Nitrilases have been found that are specific for a small range of aromatic (Harper, 1976, 1977*a, b*), heterocyclic (Robinson & Hook, 1964) and aliphatic nitriles (Fukuda *et al.*, 1971, 1973; DiGeronimo & Antoine, 1976; Yamada *et al.*, 1979, 1980; Kuwahara *et al.*, 1980). Little attention has been paid to the inducibility of nitrilases but Arnaud *et al.* (1977) have claimed that the non-specific nitrilase of *Brevibacterium* strain R312 is constitutive whereas the amidase is inducible (Jallageas *et al.*, 1978). DiGeronimo & Antoine (1976), on the basis of limited evidence, suggested that the acetonitrilase of *N. rhodochrous* strain LL100-21 is inducible.

We have shown that *N. rhodochrous* strain LL100-21 is able to grow on a range of aliphatic nitriles and amides as carbon and/or nitrogen sources, and on the related acids as carbon sources (Table 1, see also DiGeronimo & Antoine, 1976). It is also able to utilize benzonitrile and benzamide as carbon and/or nitrogen sources, and benzoate as the carbon source (Table 1). On the basis of studies on oxygen uptake by intact bacteria, as well as measurements of the enzyme activities in intact cells and extracts, acetonitrile and acetamide, and benzonitrile and benzamide have been shown to be utilized by separate enzyme systems, all of which are inducible.

Acetonitrile was converted to acetamide, and acetamide to acetate by separate enzymes (acetonitrilase and acetamidase). Neither enzyme was induced by growth on acetate but both were induced by growth on acetonitrile or acetamide. This suggests that acetonitrilase and acetamidase might be formed from structural genes on the same operon or regulon; if so, acetamide is the most likely inducer.

Unlike acetamidase and acetonitrilase, the induction of benzonitrilase, which probably converts benzonitrile directly to benzoate, appears to be separately regulated in *N. rhodochrous* strain LL100-21 from the benzamidase, which is induced by growth on benzamide. Benzonitrile was converted by *Nocardia* sp. NCIB 11216 (Harper, 1976, 1977*a*) and *Fusarium solani* (Harper, 1977*b*) directly to benzoate and ammonia. However, in contrast to our results with the *N. rhodochrous* species used in the present study, Harper (1977*a*) was only able to obtain extremely slow growth of *Nocardia* sp. NCIB 11216 on benzamide; benzamide-grown bacteria had no benzonitrilase activity.

Harper (1976, 1977*a*) found a slow activation of purified benzonitrilase of *Nocardia* sp. NCIB 11216 by benzonitrile. This process was concomitant with association of the 12 subunits of the enzyme into the oligomeric form. Preliminary experiments on the benzonitrilase of the *Nocardia* species used in the present investigation showed a time-dependent activation of the enzyme by benzonitrile, suggesting that it, too, may be oligomeric. However, a similar lag was noticed with intact bacteria for oxygen uptake and ammonia formation from benzonitrile (benzonitrile-grown bacteria) or from benzamide (benzamide-grown bacteria). This suggests that the lag may also be either due to a similar association process in the cells or to a requirement for transport of the nitrile or amide into the bacteria.

The formation of acetamide and acetate in the medium during growth of *N. rhodochrous* strain LL100-21 on acetonitrile suggested that the rate of assimilation of acetate was slower than the rate of conversion of acetamide to acetate, which in turn was less than the rate of formation of acetamide from acetonitrile. Measurements of acetonitrilase and acetamidase activities of intact bacteria and extracts (Table 3) confirmed that the latter enzyme was less active. In its

natural habitat the bacterium might not be exposed to high concentrations of acetonitrile and, if the K_m of acetonitrilase is higher than that of acetamidase, a lower relative activity of acetonitrilase would occur, resulting in little or no excretion of acetamide. Confirmation of this point awaits purification and characterization of the enzymes. DiGeronimo & Antoine (1976) also noted acetamide and acetate accumulation in the medium during growth of *N. rhodochrous* strain LL100-21 on acetonitrile, and Harper (1977a) showed benzoate production in the medium of benzonitrile-grown *Nocardia* sp. NCIB 11216.

The excretion of acetamide and acetate during growth of *N. rhodochrous* strain LL100-21 is also apparently wasteful of resources since in the natural environment they might be consumed by competing organisms. The reasons for their extrusion are not clear. Acetonitrilase and acetamidase could be located on the outside surface of the cytoplasmic membrane or be periplasmic, and hence readily accessible to substrates outside the bacterium, releasing the products into the medium. The finding that acetonitrilase and acetamidase were present in the supernatant fraction of extracts suggests that they are not bound to the membrane; further research is in progress to determine their exact cellular location.

An alternative possibility is that the bacterium is permeable to acetonitrile and acetamide. As far as we are aware, nothing is known about the permeability of micro-organisms to aliphatic nitriles. However, it might be expected that small, uncharged nitriles, with their low hydrogen-bonding capacity, would very readily permeate living cells (Stein, 1967). Brammar *et al.* (1966) suggested that acetamide and other aliphatic amides were taken up by *P. aeruginosa* via a constitutive transport system. Subsequently Betz & Clarke (1972) found that the amidase of *P. aeruginosa* was not cryptic and concluded that the organism was freely permeable to a range of amides. Farin (1976; quoted by Clarke, 1980) considered that the rate-limiting step for the uptake of acetamide was the enzyme itself and that the entry of amides was by passive diffusion.

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